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(71) Applicant (*for all designated States except US*): THE GOVERNMENT OF THE UNITED STATES OF AMERICA, asrepresented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; National Institutes of Health, Office of Technology Transfer, Box 13, 6011 Executive Boulevard, Rockville, MD 20852 (US).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): LUYTEN, Frank, P. [BE/US]; 10 Pitt Court, Rockville, MD 20850 (US). MOOS, Malcolm, Jr. [US/US]; 8507 Hazelwood Drive, Bethesda, MD 20814 (US). CHANG, Steven, Chao-Huan [-/US]; 19B Village Green North, Riverside, RI 02915 (US).

(74) Agent: ALTMAN, Daniel, E.; Knobbe, Martens, Olson & Bear, Suite 1600, 620 Newport Center Drive, Newport Beach, CA 92660 (US).

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(57) Abstract

The invention discloses extracts of cartilage tissue that exhibit chondrogenic activities *in vivo*. Proteins having chondrogenic activity are referred to as "cartilage-derived morphogenetic proteins" (CDMPs) and are disclosed to be members of the TGF-β superfamily. Methods for the preparation of extracts having biological activity are presented. Also disclosed are polynucleotides encoding two members of the CDMP family of proteins. Recombinant CDMP-1 protein was shown to have chondrogenic activity *in vivo*. Significantly, cloned polynucleotides encoding CDMP proteins from a variety of species exhibit remarkable conservation at the amino acid sequence level.

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CARTILAGE-DERIVED MORPHOGENETIC PROTEINS

Field of the Invention

The present invention relates generally to the field of cartilage and bone development. More specifically, the invention relates to cartilage-derived morphogenetic proteins that stimulate development and repair of cartilage *in vivo*.

Background of the Invention

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily that can induce endochondral bone formation in adult animals. This superfamily includes a large group of structurally related signaling proteins that are secreted as dimers and then cleaved to result in biologically active carboxy terminal domains of the proteins. These bioactive proteins are characterized by 7 highly conserved cysteine residues. Interestingly, these proteins have different roles at various stages of embryogenesis and in adult animals. Recombinant BMPs are now available and have been shown to induce endochondral bone formation when assayed *in vivo*.

Indeed, the initial discovery of the BMPs was facilitated by such *in vivo* assays for cartilage and bone development. These assays were based on the observation that bone development could be initiated by subcutaneous or intramuscular implantation of compositions comprising an extract of demineralized bone and residual bone powder. The novel proteins identified in the extracts were termed "bone morphogenetic proteins." These proteins were subsequently classified as members of the TGF-β superfamily by virtue of amino acid sequence relatedness. Screening of genomic and cDNA libraries led to the isolation of polynucleotides encoding BMP-2, -3, -4, -5, -6 and -7.

One deficiency of the bone induction assay regards its inability to distinguish the physiological roles of different BMP family members. The cartilage and bone inducing activity of the BMPs is remarkable because the normal stages of endochondral bone formation that occur during ontogeny are recapitulated in the adult animal. These stages include mesenchymal condensation, cartilage and bone and bone marrow formation and eventual mineralization to produce mature bone.

Several observations suggest that BMPs have wide-ranging extraskeletal roles in development. First, localization studies in both human and mouse tissues have demonstrated high levels of mRNA expression and protein synthesis for various BMPs in kidney (BMPs -3, -4, -7), lung (BMPs -3, -4, -5, -6), small intestine (BMPs -3, -4, -7), heart (BMPs -2, -4, -6, -7), limb bud (BMPs -2, -4, -5, -7) and teeth (BMPs -3, -4, -7). Second, several members of the family, including BMP-4 and -7, are key molecules in epithelial-mesenchymal interactions, for instance during odontogenesis. Third, BMP-2 and BMP-4 are involved in the signaling pathway that controls patterning in the developing chick limb and BMP-4 is a ventralizing factor in early *Xenopus* development. Fourth, *Drosophila* homologs of the BMPs, the decapentaplegic (dpp) and

60 A gene products, have the capacity to induce bone in mammals whereas human BMP-4 confers normal embryonic dorso-ventral patterning in *Drosophila* transformants defective in dpp expression. Thus, the BMPs are now appreciated as pleiotropic cytokines.

Interestingly, none of the known BMPs are strongly expressed in the chondroblasts and chondrocytes of the cartilage core of developing long bones. The hypertrophic chondrocytes, where both Vgr-1 (BMP-6, (Lyons et al., *Development* **109**:833 (1990)) and OP-1 (BMP-7)(Vukicevic et al., *Biochem. Biophys. Res. Commun.* **198**:693 (1994)) have been found are exceptions in this regard.

Summary of the Invention

One aspect of the present invention is a purified cartilage extract that stimulates local cartilage formation when combined with a matrix and implanted into a mammal. This extract can conveniently be produced by a method which includes the steps of: obtaining cartilage tissue; homogenizing the cartilage tissue in the presence of chaotropic agents under conditions that permit separation of proteins from proteoglycans; separating the proteins from the proteoglycans and then obtaining the proteins. The step for separating the proteins from the proteoglycans can be carried out using a sepharose column. The extract can also be obtained by additionally including the steps of separating the proteins on a molecular sieve and then collecting the proteins having molecular weights in the 30 kDa to 60 kDa size range. Articular cartilage or epiphyseal cartilage can be used in the preparation of this purified extract.

A second aspect of the present invention is a method of preparing a partially purified articular cartilage extract having chondrogenic activity. This method includes the steps of first obtaining cartilage tissue; homogenizing the cartilage tissue in the presence of chaotropic agents under conditions that permit separation of proteins from proteoglycans; separating the proteins from the proteoglycans and finally obtaining the proteins. The separation of proteins and proteoglycans can be accomplished using a sepharose column. In particular, the step for separating proteins from proteoglycans can include isolating the proteins that bind heparin Sepharose in the presence of 0.15 M NaCl but not in the presence of 1 M NaCl. An additional step in the purification procedure can include separating the proteins on a molecular sieve and then obtaining the proteins having molecular weights between 30 kDa and 60 kDa.

A third aspect of the present invention is an isolated DNA molecule that encodes a protein having chondrogenic activity *in vivo* but substantially no osteogenic activity *in vivo*. More particularly, this aspect of the invention regards a molecule having a nucleotide sequence that can hybridize to a polynucleotide which has the nucleotide sequence SEQ ID NO:11 or SEQ ID NO:12 at 55°C with 0.4× SSC and 0.1% SDS. The proteins encoded by such DNA molecles can have the amino acid sequences of SEQ ID NO:13 or SEQ ID NO:14.

A forth aspect of the present invention is a recombinant protein having chondrogenic activity *in vivo* but substantially no osteogenic activity *in vivo*. This protein can have the amino acid sequence of SEQ ID NO:13 or SEQ ID NO:14.

A fith aspect of the present invention is a method of stimulating cartilage formation in a mammal. This method includes the steps: supplying cartilage-derived morphogenetic proteins having *in vivo* chondrogenic activity; mixing the partially purified proteins with a matrix to produce a product that facilitates administration of thet partially purified proteins and implanting this mixture into the body of mammal to stimulate cartilage formation at the site of implantation. The partially purified cartilage-derived morphogenetic proteins can be obtained from either articular cartilage or epiphyseal cartilage. The matrix can also include non-cellular material. Viable chondroblast or chondrocytes can also be included in the mixture prior to implantation. The mixture can be implanted either subcutaneously or intramuscularly.

A sixth aspect of the present invention is a composition that can be administered to a mammal for the purpose of stimulating chondrogenic activity at the site of administration without substantially stimulating osteogenic activity. This composition comprises at least one cartilage-derived morphogenetic protein and a matrix. The cartilage-derived morphogenetic protein can be derived from an extract of either articular cartilage or epiphyseal cartilage. In another embodiment, the cartilage-derived morphogenetic protein is a recombinant protein. This recombinant protein can have the amino acid sequence of either SEQ ID NO:13 or SEQ ID NO:14. The matrix used to create the composition can be either fibrin glue, freeze-dried cartilage, collagens or the guanidinium-insoluble collagenous residue of demineralized bone. Alternatively the matrix can be a non-resorbable matrix such as tetracalcium phosphate or hydroxyapatite.

Brief Description of the Figures

Figure 1 presents the nucleotide and predicted amino acid sequence encoded by the full length human CDMP-1 cDNA.

Figure 2 presents the nucleotide and predicted amino acid sequence encoded by the bovine CDMP-2.

Figure 3 presents the genetic maps of chromosome 2 showing the localization of CDMP-1. The map on the right is based on the data from two separate crosses.

Figure 4 shows an alignment of segments from predicted CDMP amino acid sequences in standard one letter code.

Detailed Description of the Invention

We discovered that partially purified extracts of newborn calf articular cartilage contained an activity that induced cartilage formation when implanted subcutaneously in rats. This biological activity was reminiscent of that which characterized the BMPs. Degenerate oligonucleotide primer sets derived from the highly conserved carboxy-terminal region of the BMP family were employed

in reverse transcription-polymerase chain reactions (RT-PCR) using poly(A)$^+$ RNA from articular cartilage as a template. These procedures allowed us to determine which BMPs were expressed in chondrocytes.

Two novel members of the TGF-β superfamily were identified and designated Cartilage-Derived Morphogenetic Protein-1 (CDMP-1), and -2 (CDMP-2). The C-terminal TGF-β domains of these proteins were 82% identical, thus defining a novel subfamily most closely related to BMP-5, BMP-6 and osteogenic protein-1. Northern analyses showed that postnatally both genes were predominantly expressed in cartilaginous tissues. *In situ* hybridization and immunostaining of sections from human embryos showed that CDMP-1 was predominantly found at the stage of precartilaginous mesenchymal condensation and throughout the cartilaginous cores of the developing long bones. CDMP-2 expression was restricted to the hypertrophic chondrocytes of ossifying long bone centers. Neither gene was detectable in the axial skeleton during human embryonic development. The cartilage-specific localization pattern of these novel TGF-β superfamily members, which contrasts with the more ubiquitous presence of other BMP family members, suggested a role for these proteins in chondrocyte differentiation and growth of long bones.

The discovery of a novel subfamily of cartilage derived morphogenetic proteins suggested the existence of morphogens that primarily functioned in the induction and maintenance (i.e., balancing cartilage and bone formation at articular surfaces) of cartilaginous and bony tissues. This subfamily may also include key molecules that govern bone marrow differentiation.

The cartilage-derived morphogenetic proteins contained in the cartilage extract of the present invention, and the recombinant CDMP-1 and CDMP-2 proteins described herein are contemplated for use in the therapeutic induction and maintenance of cartilage. For example, local injection of CDMPs as soluble agents is contemplated for the treatment of subglottic stenosis, tracheomalacia, chondromalacia patellae and osteoarthritic disease. Other contemplated utilities include healing of joint surface lesions (e.g. temporomandibular joint lesions or lesions induced posttraumatically or by osteochondritis) using biological delivery systems such as fibrin glue, freeze-dried cartilage grafts, and collagens mixed with CDMPs and locally applied to fill the lesion. Such mixtures can also be enriched with viable cartilage progenitor cells, chondroblasts or chondrocytes. We also contemplate repair or reconstruction of cartilaginous tissues using resorbable or non-resorbable matrices (tetracalcium phosphate, hydroxyapatite) or biodegradable polymers (PLG, polylactic acid/polyglycolic acid) coated or mixed with CDMPs. Such compositions may be used in maxilofacial and orthopedic reconstructive surgery. Finally, the CDMPs disclosed herein hav utility as growth factors for cells of the chondrocyte lineage *in vitro*. Cells expanded *ex vivo* can be implanted into an individual at a site where chondrogenesis is desired.

We also anticipate the polynucleotides disclosed herein will also have utility as diagnostic reagents for detecting genetic abnormalities associated genes encoding CDMPs. Diagnostic testing could be performed prenatally using material obtained during amniocentesis. Any of several genetic screening procedures could be adapted for use with probes enabled by the present invention. These procedures include restriction fragment length polymorphism (RFLP), ligase chain reaction (LCR) or polymerase chain reaction (PCR).

We began our investigations by considering whether there were differences between the chondrogenic/osteogenic differentiation factors that characterized calcifying (epiphyseal, scapular cartilage) and non-calcifying (articular, nasal septum) cartilage tissues. It had been previously established that tail tendon, achilles tendon, cartilage and skin matrices were devoid of chondrogenic/osteogenic activity (originally described as "transforming potency") as measured in an *in vivo* subcutaneous implantation model in rats (Reddi A.H., 1976, "Collagen and Cell differentiation" in *Biochemistry of Collagen*, eds. Ramachandran G.N. and Reddi, A.H., pp449-478, Plenum Press, New York and London.).

We confirmed the absence of chondrogenic or osteogenic activity in crude 4 M guanidine HCl (GdnHCl) extracts of cartilage matrices, but unexpectedly discovered *in vivo* chondrogenic activity in the 0.15 M NaCl eluate of the cartilage extracts after ion exchange chromatography. The development of a unique extraction procedure (1.2 M GdnHCl and 0.5% CHAPs) followed by a heparin Sepharose affinity chromatography step confirmed the presence of *in vivo* chondrogenic activity in cartilaginous tissues. This was especially true in bovine articular and epiphyseal cartilage. When the bioactive heparin Sepharose eluates (1M NaCl eluate) were further purified using previously established procedures, molecular sieve chromatography and Con A affinity chromatography steps followed by SDS polyacrylamide gel electrophoresis and gel elution, chondrogenic activity was established. Implantation of 0.5 to 1 μg gel eluted material resulted in *in vivo* chondrogenesis. Surprisingly, and in contrast to the bone matrix purified activity, none of the peptide sequences that were found in tryptic digests of the highly purified cartilage extracts corresponded to any of the known BMPs. However, the biological activity present in the extracts was reminiscent of BMP-like activity by virtue of its loss of activity upon reduction and alkylation, its affinity for heparin Sepharose and Con A.

Although other materials and methods similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. General references for methods that can be used to perform the various nucleic acid manipulations and procedures described herein can be found in *Molecular Cloning: A Laboratory Manual* (Sambrook et al. eds. Cold Spring Harbor Lab Publ. 1989) and *Current Protocols in Molecular Biology* (Ausubel et al. eds., Greene Publishing Associates and Wiley-Interscience 1987). The disclosures contained in these references are hereby incorporated by

reference. A d scription of the experiments and results that led to the creation of the present invention follows.

We initially discovered that an extract of cartilage possessed a unique chondrogenic activity. In particular, we discovered that newborn articular cartilage contained chondrogenic activities when assayed in the *in vivo* subcutaneous implantation model. Using a procedure adapted from that used for the isolation of BMPs from demineralized bone matrix, we partially purified this activity and thereby provided evidence for the presence of BMP-like molecules in cartilage.

Example 1 describes the biochemical methods used to characterize a chondrogenic activity present in bovine cartilage.

Example 1

Characterization of Cartilage Derived

Chondrogenic Activity

Articular (metatarsophalangeal joints), scapular and nasal cartilage (300 grams wet weight per tissue) were prepared from newborn calves. Epiphyseal cartilage was dissected from fetal bovine femurs (7-8 months). The tissues were finely minced and homogenized with a Polytron (top speed, 2 x 30 seconds) in 20 volumes of 1.2 M GdnHCl,0.5% CHAPS, 50 mM Tris-HCl pH 7.2, containing protease inhibitors and extracted overnight at 4°C as described by Luyten et al., in *J. Biol. Chem.* **264**:13377 (1989), which is hereby incorporated by reference. The disclosure of this article is hereby incorporated by reference. Sorgente et al., (*Biochem Biophys. Acta.* **282**:441 (1972)) disclosed these procedures extract >90% of the lower molecular weight matrix while leaving most of the high molecular weight proteoglycans behind. The extracts were concentrated and exchanged with 6 M urea by diafiltration using an Ultrasette™ (Filtron Technology Inc., MA) and loaded on a 0.5L heparin Sepharose (Pharmacia/LKB, NJ) column. Thereafter, the column was washed with 5 bed volumes of 6 M urea, Tris HCl pH 7.4 with 0.15 M NaCl, and then eluted with 2 vol 1 M NaCl in the same buffer. Chondrogenic activity was assayed by reconstituting a portion of the eluate with 25 mg of guanidine-insoluble collagenous residue of demineralized rat bone matrix according to procedures described by Luyten et al., in *J. Biol. Chem.* **264**:13377 (1989). Implants were recovered after 10 days and alkaline phosphatase activity was measured as a biochemical indicator of cartilage and/or bone formation. The specific activity was expressed as units of alkaline phosphatase/mg of protein used for reconstitution in the bioassay. Implants were also examined histologically for evidence of cartilage formation using standard procedures known to those of ordinary skill in the art.

Additional purification steps were also performed. The 1 M NaCl eluate of articular cartilage, which contained biological activity, was concentrated by diafiltration and loaded onto a Sephacryl S-200 HR gel filtration column (XK 50/100, Pharmacia/LKB, NJ). After molecular

sieve chromatography, the bioactive fractions were pooled and exchanged with 50 mM Hepes, pH 7.4, containing 0.15 M NaCl, 10 mM $MgSO_4$, 1mM $CaCl_2$ and 0.1% (w/v) CHAPS using Macrosep™ concentrators (Filtron Technology Inc., Northborough, MA). The equilibrated sample was mixed with 1 ml Con A Sepharose (Pharmacia-LKB) previously washed with 20 volumes of the same buffer according to the procedure described by Paralkar et al., in *Biochem. Biophys. Res. Comm.* **131**:37 (1989). After overnight incubation on an orbital shaker at 4°C, the slurry was packed into a disposable 0.7 cm ID Bio-Rad column and washed with 20 volumes of the Hepes buffer to remove unbound proteins. Bound proteins were eluted with 20 volumes of the same buffer containing 500 mM methyl-D-mannopyronaside. The eluate was concentrated to 200 µl using Macrosep™ concentrators. Macromolecules were then precipitated overnight with 9 volumes of absolute ethanol at 4°C. The precipitate was redissolved in 1 ml 6 M urea, Tris HCl pH 7.4. The bioactive bound protein was then mixed with 2 X Laemmli sample buffer (without reducing agents) and electrophoresed on a 12% preparative SDS/polyacrylamide gel. Gel elution of the separated protein fractions and testing for biological activity was performed as described by Luyten et al., in *J. Biol. Chem.* **264**:13377 (1989). We also observed that, after reduction with dithiothreitol and alkylation with iodoacetamide, substantially all of the cartilage-forming activity contained in the protein sample was lost.

Results indicated that each of the crude extracts of the different cartilaginous tissues (articular, nasal, scapular or epiphyseal) were inactive when tested directly in the *in vivo* cartilage and bone inducing assay. This finding confirmed previously described results published by Reddi in "Collagen and Cell differentiation" in *Biochemistry of Collagen* (eds. Ramachandran G.N. and Reddi, A.H., pp449-478, Plenum Press, New York and London (1976)). However, after heparin affinity chromatography (Sampath et al., *Proc. Natl. Acad. Sci. U.S.A.* **84**:7109 (1987)), chondrogenic activity was recovered in the 1 M NaCl eluate from articular cartilage extracts. An additional molecular sieve chromatography step (S200) was required to recover chondrogenic activity from epiphyseal cartilage extracts. Similar results were obtained upon ion exchange chromatography using DEAE Sephadex (0.15 M NaCl eluate). Significantly, no activity was detected in the extracts of the other cartilaginous tissues.

The highest specific activity was obtained for material derived from articular cartilage (1 U alkaline phosphatase/mg protein). This material was used for characterization of the bioactivity. Further purification of the active fraction by molecular sieve chromatography on Sephacryl S-200HR (specific activity 112 U/mg), and affinity chromatography on Concanavalin A (specific activity 480 U/mg), established the presence of cartilage and bone inducing activity characteristic of the members of the BMP family. Gel lution experiments with the Con A bound bioactive fraction demonstrated that the activity resided between roughly 34 and 38 kDa (specific activity of the gel eluted fractions was 2143 U/mg). We have also demonstrated that size separation by

molecular sieve chromatography can be used to purify biological activity in the 30-60 kDa size range. In addition, loss of activity that was observed following reduction and alkylation suggested that the bioactivity was induced either by a known or a new member(s) of the BMP family.

Given the demonstration that cartilage contained a BMP-like activity, we proceeded to isolate polynucleotides encoding the responsible proteins. Specifically, degenerate primers corresponding to conserved regions of known BMPs were designed. These primers were then employed to amplify polynucleotides using reverse transcribed mRNA from articular cartilage as a template. These procedures ultimately led to the identification of two novel cDNAs, which we called CDMP-1 and CDMP-2.

Example 2 describes the methods used to amplify polynucleotides corresponding to mRNAs that were expressed in cartilage tissue and that exhibited at least weak sequence similarity to conserved regions of the BMP mRNAs.

Example 2

PCR Amplification of cDNAs Encoding Cartilage-Derived Morphogenetic Proteins

Total RNA from bovine articular chondrocytes (metatarsophalangeal joints) was extracted using a modified acid guanidine-phenol-chloroform method described by Chomczynski et al., in *Anal. Biochem.* **162**:156 (1987) and by Luyten et al., in *Exp. Cell. Res.* **210**:224 (1994). Poly(A)$^+$ RNA was isolated using magnetic beads (PolyATract™, Promega, Madison, WI). Four degenerate oligonucleotide primers corresponding to highly conversed motifs in the C-terminal region of the BMPs were used; S1: 5'-GGITGG(C/A)AIGA(C/T)TGGAT(A/C/T)(A/G)TIGC(A/C/G/T)CC-3' (SEQ ID NO:1) corresponding to amino acids [GW(Q/N)DWI(I/V)AP] (SEQ ID NO:2); S2: 5'-GGITGG(A/T)(G/C)(I)GA(G/A)TGGAT(T/C/A)ATI(A/T)G(A/C/G/T)CC-3' (SEQ ID NO:3) corresponding to amino acids [GWSEWIISP] (SEQ ID NO:4); AS1: 5'-A(A/G)(A/G)GT(C/T)TG(A/C/G/T)AC(A/G)AT(A/G)GC(A/G)TG(A/G)TT-3' (SEQ ID NO:5) corresponding to amino acids [NHAIVQTL] (SEQ ID NO:6); AS2:5'-CAI(C/G)C(A/G)CAI(G/C)(A/C/T)I(C/T)(C/G/T)IACIA(C/T)CAT-3' (SEQ ID NO:7) corresponding to amino acids [M(V/I)V(E/R)(G/S/A)C(G/A)C] (SEQ ID NO:8). Nucleotides in parenthesis denote sites of degeneracy and I denotes inosine. First strand cDNA synthesis was performed using 1 μg Poly(A)$^+$ or 5 μg total RNA with oligo dT, random hexanucleotide primers, or the antisense degenerate primers, AS1 and AS2. Successful PCR amplifications were performed with the degenerate sense primers, S1 or S2 in combination with the AS1 antisense primer were performed using conditions described by Wharton et al., in *Proc. Natl. Acad. Sci. U.S.A.* **88**:9214 (1991). The reaction products were electrophoresed on 1.2% agarose gels, and DNA fragments of appropriate sizes were excised and purified using the Magic PCR Prep DNA purification system (Promega, Madison, WI). Reamplification was performed with the same primers and each PCR

product was subcloned into the PCR II vector using the TA Cloning™ System (InVitrogen Corporation, San Diego, CA). Results of RT-PCR using poly(A)$^+$ RNA isolated from newborn bovine articular cartilage as template and sets of degenerate oligonucleotide primers (S1/AS1 and S1/AS2) yielded amplification products of 120 bp and 280 bp.

Subcloned inserts were sequenced according to the dideoxy DNA sequencing method of Sanger et al., (*Proc. Natl. Acad. Sci. U.S.A.* 74:5463 (1977)). Both DNA strands were sequenced using Sequenase Version 2.0 DNA polymerase according to manufacturer's instructions (USB, Cleveland, OH) with at least two-fold redundancy. Confirmatory data in ambiguous regions were obtained by automated thermal cycle sequencing with an Applied Biosystems Model 370A sequencer and by using 7-deaza-GTP (USB, Cleveland, OH). The sequencing data were obtained from restriction fragments subcloned into pBluescript (Stratagene, La Jolla, CA) using either M13 forward and reverse primers or synthetic oligonucleotide primers.

The results from a computer-assisted search of the nucleic acid sequence databases indicated the cloned inserts encoded BMP-2, -6, BMP-7 (OP-1), and several other BMP-like sequences. Identification of these latter gene fragments led us to isolate larger cDNAs that included the entire protein coding region of the transcript. The availability of such clones facilitated both a more precise analysis of the encoded BMP-like protein and permitted studies aimed at localizing the expression of these genes. Thus, cloned inserts having novel BMP-like sequences were isolated, radiolabeled and used to screen both human and bovine articular cartilage cDNA libraries.

Example 3 describes the methods used to isolate human and bovine cDNAs that corresponded to a segment of one of the BMP-like gene segments that were amplified from cartilage mRNA templates.

Example 3

Library Screening

A 120 bp PCR fragment encoding part of the C-terminal domain of novel BMP like genes (dashed line, Figure 1) was used to screen two cDNA libraries. One library, from adolescent human articular cartilage poly(A)$^+$ RNA (kindly provided by Dr. Björn Olsen, Harvard, Boston, MA), was primed with oligo dT and constructed in the λgt11 vector. The other was a bovine oligo dT and random primed articular cartilage cDNA library constructed in the UNIZAP®XR vector (Stratagene, La Jolla, CA). Approximately 1 x 10^6 plaques from each library were screened by standard procedures. Hybridizations were performed for 20 hours at 42°C in 6 x SSC, 1 x Denhardt's solution, 0.01% tRNA, 0.05% sodium pyrophosphate and the membranes (DuPont 137 mm nylon membranes, New England Nuclear, MA) were washed to final stringency of 6 x SSC, 0.1% SDS at 55°C for 20 minutes.

Thus, cloned inserts having novel BMP-like sequences were isolated, radiolabeled and used to screen both human and bovine articular cartilage cDNA libraries. Six clones were isolated from the human cDNA library. The sizes of the EcoRI inserts (2.1 kb) and their restriction maps were found to be identical for all six clones. One clone was used for nucleotide sequencing. An open reading frame encoding a BMP related protein, designated CDMP-1, was identified. It appeared that the human cDNA clone lacked the coding region for the first methionine and signal peptide. The 5' end of the human CDMP-1 was subsequently obtained from a human genomic clone isolated from a library constructed in the EMBL-3 vector (Clontech, Palo Alto, CA). The 5' end of human CDMP-1 contained a consensus translation initiation sequence disclosed by Kozak (*J. Biol. Chem.* **266**:19867 (1991)) immediately followed by a putative transmembrane signal sequence described by Von Heijne (*Nucl. Acids Res.* **14**:4683 (1986)). The nucleotide sequence and the translation of the open reading frame of CDMP-1 are presented in Figure 1. As shown in the figure, the CDMP-1 protein was predicted to have 500 amino acids, to consist of a pro-region of 376 amino acids, a typical cleavage site (Arg-Xaa-Xaa-Arg/Ala) (SEQ ID NO:9), and a C-terminal domain of 120 amino acids containing the seven highly conserved cysteines characteristic of the TGF-β gene family. A single N-linked glycosylation site is located in the pro-region (marked by an asterisk in the figure). A putative signal peptide is underlined in bold. A termination codon (TGA) is shown in the 5' untranslated region. The bold dashed underline indicates the fragment obtained by RT-PCR that was subsequently used to screen cDNA libraries. The 13 amino acid peptide used to raise polyclonal antibodies in rabbits is underlined. A vertical arrowhead marks the boundary between the sequence obtained from genomic DNA and cDNA.

Two clones with inserts of 2.8 kb were isolated from a bovine articular cartilage cDNA library. Both clones were sequenced and the open reading frame was found to encode another novel TGF-β related protein, designated CDMP-2. The CDMP-2 cDNA and predicted protein sequences are presented in Figure 2. As shown in the figure, the open reading frame contained a putative proteolytic processing site (boxed), preceding a 120 amino acid mature C-terminal region containing seven highly conserved cysteines. The 5' end with the first methionine and signal peptide were missing. The product obtained by RT-PCR (bold dashed underline) was used to screen a bovine cDNA articular cartilage library. The ApaI sites used to release a cDNA fragment for hybridization experiments are underlined. At the 5' end, the pro-region lacked the first methionine and signal peptide. The mature C-terminal domain of 120 amino acids showed 82% identity with CDMP-1.

Alignment of the carboxy terminal domains of CDMP-1 and -2 with other members of the BMP family revealed an amino acid identity of about 50% with BMP-5, BMP-6 and OP-1 (BMP-7). These results suggested that CDMP-1 and CDMP-2 are members of a new subfamily.

The amino acid sequence similarity between the human CDMP-1 and bovine CDMP-2 proteins prompted us to further investigate conservation of the CDMPs across different species. In particular, we employed a PCR amplification protocol to isolate CDMP cDNA sequences from a variety of species. Based on alignments of the predicted proteins encoded by these cDNAs, we identified a highly conserved amino acid sequence spanning 31 residues. Only 5 amino acid positions within this sequence showed variability. All remaining positions were identical for all isolates. As disclosed in the following Example, even the 5 variable positions showed a high degree of conservation. This structural conservation likely represents a functional domain that is characteristic of the CDMP family of proteins. Those of ordinary skill in the art will appreciate that such extraordinary amino acid sequence conservation is indicative of a functional domain. We therefore believe the consensus amino acid sequence presented in the following Example is critical to the biological activity of the CDMPs.

Example 4 describes the procedures used to identify an amino acid consensus sequence that characterizes the CDMPs from several different species.

Example 4

Identification of a Highly Conserved Consensus Sequence in CDMP Proteins

RNA isolated from chicken sternal cartilage, bovine articular cartilage and human articular cartilage was employed as the template in RT-PCR protocols using the primers S1 and AS1 and procedures described under Example 2. Genomic DNA isolated from Xenopus and zebrafish was also used as the template for amplification of related gene sequences in a PCR protocol that employed the same primer sets. Amplified DNA fragments were subcloned according to standard procedures. The inserts from various isolates were sequenced by standard dideoxy chain termination protocols. Aligned segments of the predicted proteins encoded by the cloned cDNAs are presented in Figure 4.

Results of the protein alignments clearly indicated that CDMP family members from several species shared a common amino acid sequence motif in the region of the proteins encoded by the amplified cDNA segments. Of the 31 amino acid positions presented in Figure 4, all but 5 were occupied by identical amino acid residues for all of the isolates. The variable amino acids were located at positions 3, 7, 11, 16 and 18. Position 3 was occupied either by I, M or V. Position 7 was occupied by either D or E, both of which have acidic side groups. Position 11 was occupied by either Y, F or H. Position 16 was occupied by L or V, and position 18 was occupied by D or E. The consensus deduced from this alignment was:

W-I-(I/M/V)-A-P-L-(D/E)-Y-E-A-(Y/F/H)-H-C-E-G-(L/V)-C-(D/E)-F-P-L-R-S-H-L-E-P-T-N-H-A

(SEQ ID NO:15). This consensus sequence is slightly broader than the one shown in Figure 4.

as it encompasses all the variations observed in the sequenced polynucleotides. The consensus sequence in the figure indicates predominating amino acids.

We believe that biologically active CDMPs will possess this highly conserved amino acid sequence motif. Proteins having different amino acids in the variable positions in the consensus will likely represent novel family members having distinct functions. We also believe that polynucleotide hybridization probes or PCR primers designed based on this conserved protein motif can be used to isolate cDNAs encoding CDMP family members or related proteins.

Southern analyses were also carried out to investigate possible sequence conservation across species and to localize the CDMP-1 gene to a particular chromosome.

Example 5 describes the Southern blotting protocols used to detect DNA sequences corresponding to the CDMP-1 cDNA.

Example 5

Genetic Mapping of CDMP-1

Southern hybridization was performed using the evolutionary relatedness blot (Bios Laboratories, New Haven, CT) under conditions recommended by the manufacturer. The panel of EcoRI-digested genomic DNAs included human (*homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus domesticus*), frog (*Xenopus laevis*), lobster (*Homarus americanus*), mussel (*Mytilus edulis*), fish (*Tautoga onitis*), fruit fly (*Drosophila melanogaster*), nematode (*Caenorhabditis elegans*), yeast (*Saccharomyces cerevisiae*) and bacteria (*E.coli*). The 2.1 kb CDMP-1 EcoRI fragment originally obtained from the cDNA library was used as a probe, and the blot was washed to a final stringency of 0.4 x SSC, 0.1% SDS, at 55°C.

Results from these Southern analyses using the original 2.1 kb human CDMP-1 cDNA probe (starting from amino acid position 40), showed 5.9 and 2.6 kb bands in humans and strong hybridization in both mouse and chicken. Fainter bands were seen in fish, frog and lobster after 5 days autoradiographic exposure. No hybridization was detected to *Drosophila* DNA.

The 2.1 kb ApaI fragment of CDMP-1 was used as a hybridization probe on Southern blots to type mouse genomic DNAs from two genetic crosses: (NFS/N or C58/J x *M. m. musculus*) x *M. m. musculus* (see Joseph et al., *Mol. Immunol.* **30**:733 (1990)) and (NFS/N x *M. spretus*) x *M. spretus* or C58/J (see Adamson et al., *Virology* **183**:778 (1991)). DNAs from these crosses have been typed for over 650 markers including the Chr 2 markers *Snap* (synaptosomal associated protein 25), *Psp* (parotid secretory protein), *Emv15* (ecotropic murine leukemia virus 15), *Src* (src oncogene), and *Cd40* (cluster designation 40). Probes for these markers and restriction fragment length polymorphisms used to type these crosses have been described by Joseph et al., in *Mol. Immunol.* **30**:733 (1990) and by Grimaldi et al., in *J. Immunol.* **149**:3921 (1992). *Src* was typed using a mouse Src probe obtained from E. Rassart (U. Quebec, Montreal) following XbaI digestion in the *musculus* cross and BamHI digestion in the *spretus* cross.

Results from Southern blotting with the 2.1 kb cDNA described above identified EcoRI fragments of 7.1 and 2.0 kb in *M. spretus* and *M. m. musculus* and 6.8 and 3.2 kb in NFS/N and C58/J.

Inheritance of the polymorphic fragments in the progeny of the two crosses used for mapping was compared with inheritance of over 650 markers previously mapped to all 19 autosomes and the X chromosome. The gene encoding CDMP-1 was found to be linked to markers on Chr 2 just proximal to Src. The closest linkage was observed with *Psp* and *Emv15*. No recombination was observed between Cdmp1 and Psp in the 100 mice typed for both markers indicating that these genes are within 3.0 cM at the 95% confidence level. Similarly, the absence of recombination between Gdf5 (Storm et al., *Nature* 368:639 (1994)) and Cdmp1 in 125 mice suggested these genes colocalized within 2.4 cM. This map location suggested close proximity to the brachypodism locus (bp). A genetic map that presents the localization of CDMP-1 on chromosome 2 is shown in Figure 3. Recombination fractions are given to the right of each map of the diagram for each adjacent locus pair or cluster. Numbers in parenthesis represent the percent recombination and standard error calculated as described by Green in Genetics and Probability in Animal Breeding Experiments, Oxford University Press, New York (1981). The map on the left is an abbreviated version of the Chr 2 Committee Map disclosed by Siracusa et al., in *Mammal Genome* 4:S31 (1993), and shows the map location of *bp* relative to the other markers typed in the crosses used here.

The brachypodism (*bp* mice) disorder is characterized by a distinct shortening of the limbs without other tissue abnormalities. The defect has previously been attributed to lack of production of a chondrogenic signal by mesenchymal cells at the time of chondrogenesis (Owens et al., *Dev. Biol.* 91:376 (1982)). During the course of our investigation, an independent study by Storm et al. (*Nature* 368:639 (1994)) described the isolation of the mouse CDMP-1 homolog, called Gdf-5, and established its linkage to the bracypodism (*bp*) mutation. The types of mutations observed in bp mice were found to be effective null-mutations for the gene encoding Gdf-5/CDMP-1. The pattern of expression of CDMP-1 throughout the cartilaginous core observed during human embryonic long bone development, coupled with the *bp* mutation in mice, indicated that its primary physiological role was most likely at the stage of early chondrogenesis and chondrocyte differentiation in the developing limb.

The foregoing results indicated the CDMP-1 and CDMP-2 cDNAs were novel, exhibited moderate sequence conservation across species as judged by evolutionary hybridization studies and that the CDMP-1 gene localized to mouse chromosome 2. We proceeded to examine the pattern of CDMP expression at the mRNA level.

Example 6 demonstrates the methods used to determine the pattern f CDMP mRNA expression.

Example 6

CDMPs are Predominantly Expressed in Cartilage During Postnatal Life

Equal amounts of poly(A)$^+$ RNA (2 μg) from bovine criocoid and articular cartilage were electrophoresed on 1.2% agarose-formaldehyde gels and then transferred to Nytran membranes (Schleicher and Schuell, Kenne, NH) according to standard laboratory procedures. Multiple Tissue Northern blots were obtained from Clontech (Palo Alto, CA). The membranes were prehybridized for 3 hours at 42°C in hybridization buffer (5 x SSPE, 5 x Denhardt's solution, 50% formamide, 1% SDS and 100 μg/ml freshly denatured salmon sperm DNA). Hybridizations with [^{32}P]dCTP labeled probes, having specific activities of at least 1 x 10^9 CPM/μg, were performed overnight under the same conditions as the prehybridization. Probes included the cDNA probe for human glyceraldehyde-3-phosphate dehydrogenase (1.1 kb, G3PDH (Clontech, Palo Alto, CA), an ApaI fragment (bp 470 - 1155) of CDMP-1, and an ApaI fragment (bp 194 - 677) of CDMP-2. The CDMP-1 and CDMP-2 probes were chosen to avoid the highly conserved carboxy-terminal domain, thereby minimizing the potential for cross hybridization with other members of the gene family. Following hybridization, the filters were washed to a final stringency of 55°C, 0.4 x SSC, 0.1% SDS. The mRNA expression levels were quantified using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Results from Northern analyses of a number of postnatal tissues indicated that CDMP-1 could predominantly be detected in newborn articular and cricoid cartilage. In both cases a single mRNA transcript of approximately 3 kb was observed. The CDMP-1 mRNA was not detected in pancreas, kidney, skeletal muscle, liver, lung, placenta, brain or heart. In contrast, BMP-3 and BMP-7 transcripts were detected in subsequent hybridizations of the same blots in mRNA samples from lung, kidney, brain and small intestine. This finding was consistent with previous results disclosed by Vukicevic et al., (*J. Histochem. Cytochem.* 42:869 (1994)). CDMP-2 mRNA was detected in postnatal bovine articular and cricoid cartilage as a 4.6 kb mRNA band. After prolonged exposure, weak hybridization signals were detected at 4.6 kb and 4.0 kb in mRNA from colon and small intestine, skeletal muscle and placenta.

Two other procedures were used to localize and visualize expression of the CDMP-1 and CDMP-2 gene products. These approaches relied on detection of mRNA and protein in tissue sections prepared for analysis by microscopy.

Example 7 describes the methods used to demonstrate the preferential expression of CDMPs during human embryogenesis.

Example 7

CDMPs are Preferentially Expressed in the Cartilaginous Cores of Long Bone During Human Embryogenesis

In Situ Hybridization

Tissues from human embryos were obtained after pregnancy termination at from 5 to 14 weeks of gestation. Embryo age was estimated in weeks (W) on the basis of crown-rump length (CRL) and pregnancy records of the conceptual age. They were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), embedded in paraffin, sectioned serially at 5-7 μm, and mounted on silanated slides. The tissues used in the present study were obtained from legally sanctioned procedures performed at the University of Zagreb Medical School. The procedure for obtaining the human autopsy material was approved and controlled by the Internal Review Board of the Ethical Committee at the School of Medicine, University of Zagreb and Office of Human Subjects Research (OHSR) at the National Institutes of Health, Bethesda, MD. *In situ* hybridization was done as described by Vukicevic et al., (*J. Histochem. Cytochem.* **42**:869 (1994)) and by Pelton et al. (*Development* **106**:759 (1989)). Briefly, sections were incubated overnight at 50°C in a humidified chamber in 50% formamide, 10% dextran sulfate, 4 x SSC, 10 mM dithiothreitol, 1 x Denhardt's solution, 500 μg/ml of freshly denatured salmon sperm DNA and yeast tRNA with 0.2-0.4 ng/ml ^{35}S labeled riboprobe (1 x 10^9 CPM/μg). Apal fragments of CDMP-1 and of CDMP-2 (described above) from the pro region, subcloned in both sense and anti-sense direction into pBluescript II $(SK)^+$ vector (Strategene, CA), were used as transcription templates. Riboprobes were then prepared using T7 RNA polymerase (Sure Site Kit, Novagen, Madison, WI) according to the manufacturer's instructions and used with and without prior alkaline hydrolysis. After hybridization, the sections were washed as described by Lyons et al., in *Development* **109**:833 (1990), to a final stringency of 0.1 x SSC, 65°C for 2 x 15 minutes. After dehydration through a graded ethanol series containing 0.3 M ammonium acetate, slides were covered with NTB-2 emulsion (Kodak) and exposed between 1 and 3 weeks. After development, the slides were stained with 0.1% toluidine blue, dehydrated, cleared with xylene and mounted with Permount.

Immunostaining

A polyclonal antibody to the peptide QGKRPSKNLKARC (SEQ ID NO:10) (amino acids 388-400; prepared by Peptide Technologies, Gaithersburg, MD), which belongs to the mature secreted protein of CDMP-1, was raised in rabbits. Before immunization, the peptide was conjugated to Imject^R Malemide Activated Keyhole Limpet Hemocyanin (Pierce, Rockfor, IL). Searches performed using the BLAST (Altschul et al., *J. Mol. Biol.* **215**:403 (1990)) network service available through the National Center for Biotechnology Information indicated that the

peptide does not show sequence identity with any known protein or BMP. The embryonic tissue sections were stained as recommended by the manufacturer using immunogold as a detection system (Auroprobe LM; Janssen, Belgium) and counterstained with 0.1% toluidine blue. The primary antibody (crude antiserum) was used at a concentration of 15 μg/ml in PBS with 0.5% bovine serum albumin (BSA) for 1 hour. In the controls, the primary antibody was replaced with BSA, normal rabbit serum, or secondary antibody alone.

Results indicated that, at 6 weeks of gestation, CDMP-1 transcripts were detected in precartilage condensations within the developing limbs. At 7.5 - 8.5 weeks of gestation, CDMP-1 mRNA expression was found in the cartilaginous cores of long bones, including the articular surfaces. In areas of active cartilage degradation and bone matrix formation, CDMP-1 expression was also detected in hypertrophic chondrocytes. Remarkably, no expression was detected in the axial skeleton and only low mRNA levels were observed in other tissues, such as distal convoluted tubules of the developing kidney, brain and placenta. Immunohistochemical staining indicated that CDMP-1 protein colocalized with the mRNA. However, in addition to the sites of transcription, the protein was also found in the surrounding cartilaginous matrix and in osteoblast-like cells from the primary ossification centers of long bones.

Between 9 and 10 weeks of gestation, CDMP-2 expression was predominantly localized in the more mature and hypertrophic chondrocytes in regions of invasion by blood vessels through the periosteal bony collar of the developing long bone. Again, as for CDMP-1, no hybridization was detected in the vertebral bodies in the corresponding sections and stages of human embryonic development. Low expression levels were detected in the periosteum.

The expression pattern of CDMP-2 suggests it is involved in the terminal differentiation of chondrocytes (hypertrophic and mineralizing) and at the earliest stages of endochondral bone formation, including angiogenesis and osteoblast differentiation. In addition, the relatively high levels of expression (detectable in total RNA blots) in postnatal cartilage suggest possible roles in the maintenance and stabilization of the cartilage phenotype after birth.

We have also designed experiments aimed at determining whether all of the chondrogenic activity contained in cartilage extracts can be attributed to the proteins encoded by the CDMP-1 and CDMP-2 cDNAs. Our approach involves the production and use of neutralizing antibodies using synthetic peptides or recombinant CDMP-1 and CDMP-2 proteins as immunogens. Antibodies raised against these peptides or proteins will be tested for their ability to deplete cartilage extracts of chondrogenic activity. If antibodies specific for the recombinant proteins fail to deplete the extracts of cartilage-forming activity, then residual activity will be due to factors within the extract that are separate from proteins encoded by the CDMP-1 and CDMP-2 proteins. Alternatively, if antibodies raised against the peptides or recombinant proteins can remove

cartilage-inducing activity from the extracts, this will confirm that CDMP-1 and/or CDMP-2 must be responsible for the active agents contained in the extracts.

Example 8 describes the methods that will be used to raise antibodies against synthetic peptides and recombinant CDMP-1 and CDMP-2 proteins. Antibodies produced in this fashion will be tested for their ability to deplete extracts containing CDMP activity.

Example 8

Production and Use of Anti-CDMP Antibodies

Specific monoclonal and polyclonal antibodies will be raised against peptides designed from the mature protein of the CDMPs. Preferentially, the region between the protein cleavage site and the first cysteine of the CDMP-1 and CDMP-2 proteins will be used to design the peptides. In addition, the cDNAs encoding the mature region of the CDMPs will be subcloned in the bacterial pET expression vector, and expressed as monomers in the bacterial expression system. The protein expressed in this system will be used to raise additional antibodies, and to determine the immunoreactivity of the various antisera in Western blots. The bacterially expressed monomers will be refolded into biologically active dimers using standard protocols. This approach may afford another source of recombinant protein.

The antisera obtained in this fashion will be used to further establish the synthesis of the CDMPs by chondrocytes *in vivo* and *in vitro*, and to link the cloned CDMPs to the chondrogenic activity found in cartilage extracts. Conditioned media obtained from chondrocyte cultures and partially purified chondrogenic cartilage extracts after heparin sepharose affinity chromatography, molecular sieve chromatography and Con A chromatography, will be analyzed for the presence of CDMPs by Western blot analysis. Due to the possible heterogeneity of the highly purified chondrogenic cartilage extracts, the antibodies will be used to reduce or deplete the chondrogenic/osteogenic activity in purified fractions in a standard immunoprecipitation experiment.

An important aspect of our invention regards the production and use of recombinant proteins that possess the biological activities of the CDMPs. The following Example describes methods and results that illustrate the production of recombinant CDMP-1 and CDMP-2 in transfected 293 cells, COS-1 cells, and CHO-1 cells. We discovered that 293 cells express BMP-7 that could conceivably contaminate preparations of recombinant CDMPs. To avoid possible ambiguities in the interpretation of our results, recombinant CDMP-1 produced in COS-1 cells was used to demonstrate cartilage forming activity. Although the production of recombinant CDMPs in this fashion was rather inefficient, the key finding illustrated by our results was that recombinant protein had the desired cartilage-forming activity. Unexpectedly, and in contrast to the related BMPs, recombinant CDMP-1 induced cartilage formation without noticeable bone formation.

Example 9 describes the procedures used to produce recombinant CDMP proteins. The results presented in the Example confirm that the recombinant cartilage-derived proteins stimulated cartilage formation.

Example 9

Production of Recombinant CDMPs and Assessment of Bioactivity

Full length CDMP-1 was subcloned into the mammalian expression vector pcDNA3 (Invitrogen Corporation, San Diego, CA) containing the cytomegalovirus early gene promotor and other elements required for expression in mammalian cells. COS 1 cells were cultured in Opti-MEM I (Gibco/BRL, Gaithersburg, MD) in the presence of 5% fetal bovine serum and antibiotics. The cells were grown to approximately 70-80% confluency in 150 mm dishes and transfections of the respective plasmids (20 μg plamid) were carried out by the calcium phosphate method using the transfection MBS mammalian transfection kit (Stratagene, La Jolla, CA). The cells were incubated with the calcium phosphate-DNA mixture for 3 hours at 35°C. Supernatants were removed and the plates were washed 3 times with PBS. 15 ml of Opti-MEM I (serum reduced medium) was added in the absence of serum, and the dishes were incubated overnight. Transfection efficiencies were tested by transfection of a control plasmid, pCMVβ-gal and cell extracts were assayed for β-galactosidase activity. Conditioned media were collected at 24 hour intervals for a period of 96 hours. The pooled media was centrifuged to remove cell debris and then concentrated using Mascrosep 10 concentrators (Filtron Technology Inc., Northborough, MA). Further purification of recombinantly expressed protein was performed as described in preceding Examples. In one exemplary procedure, the conditioned media was adjusted to 4 M urea, 25 mM Tris HCl (pH 7.0) and applied to a heparin Sepharose column. The column was washed with the same buffer containing 0.1 M NaCl, and eluted with 1 M NaCl. The heparin Sepharose unbound and eluted fractions were assayed for biological activity as described by Luyten et al., in *J. Biol. Chem.* **264**:13377 (1989).

Biological activity of the recombinantly expressed protein was investigated using *in vitro* and *in vivo* chondrogenic/osteogenic assays. For the *in vivo* assay, fractions containing the CDMPs were precipitated with ethanol, or dried onto a carrier such as bone matrix residue (mainly collagen type I particles) and cartilage matrix residue (cartilage tissue after extraction with chaotropic agents, and powderized to particles with a size of 75-400 μm). The dried pellet (about 25 mg) was implanted subcutaneously in rats. Implants were harvested after 11 and 21 days, and analyzed for chondrogenesis/osteogenesis using alkaline phosphatase determinations. Histological analysis of recovered sampl s was also performed using toluidine blue, alcian blue and safranin O staining.

Results obtained using the recombinant CDMP-1 produced in COS-1 cells revealed chondrogenic activity in this *in vivo* assay. Significantly, no osteogenic activity was observed in

any of the recovered samples. Osteogenic activity would ordinarily have been observed if the sam procedures had been carried out using recombinant BMPs. This difference highlighted the unique properties of recombinant CDMP-1.

Future *in vitro* chondrogenic experiments will be performed to determine the precursor cells responsive to the CDMPs. Undifferentiated (10T1/2 cells, bone marrow stromal cells, mesenchymal stem cells) and already committed skeletal cells (limb bud cells, perichondrial or periosteal cells, fetal epihyseal chondroblasts, and chondrocytes) will be transfected with the cDNAs or treated with recombinantly expressed CDMPs to evaluate the stage of differentiation associated with the chondrogenic activity of the CDMPs.

Future *in vivo* chondrogenic experiments will be directed to expression of large quantities of CDMP-1 and CDMP-2 by stable transfectants. We contemplate the use of hybrid expression constructs in which the pro-region of one BMP family member (for example BMP-2) is operationally linked to the regions encoding the mature CDMPs. We also anticipate *in vivo* assays based on implantation in other sites, apart from subcutaneous implantation, which may reveal distinct or superior biological activities of the CDMPs. For example, we anticipate implantation in the synovial cavity may have utility in such assays.

The CDMPs disclosed in the present invention have important applications in the repair of cartilage defects. We contemplate two general approaches for this type of therapy. In the first place, the CDMPs are used as lineage-specific growth factors for the *ex vivo* expansion of chondrocytes isolated from a donor who requires therapeutic intervention. Following expansion, these cells can be reimplanted into a cartilage lesion in the donor, whereafter repair of cartilage will take place. In a different scenario, CDMPs are introduced into a cartilage lesion. For example, a composition containing an appropriate CDMP or mixture of CDMPs can be implanted into a lesion for the purpose of stimulating *in vivo* chondrogenesis and repair of cartilage. The CDMPs can be combined with any of a number of suitable carriers. An appropriate carrier can be selected from the group comprising fibrin glue, cartilage grafts, and collagens. An implantable mixture can be introduced into the site of a lesion according to methods familiar to those having ordinary skill in the art. In one application, we contemplate that periosteal synovial membrane flap of tissue or inert material can be impregnated with CDMPs and implanted for cartilage repair.

Example 10 illustrates one application of the CDMP preparations described above. Specifically, the following Example describes the use of CDMPs to facilitate repair of cartilage in the knee joint.

Example 10

Treatment of Deep Knee Defects With Cartilage-Derived Morphogenetic Proteins

A young patient having a large defect in the articular surface of the knee joint is identified. According to standard surgical procedures, a periosteal flap is obtained from the bone beneath the joint surface of rib cartilage. The tissue flap is pre-soaked in an extract containing CDMPs or alternatively in a solution containing recombinant CDMPs. The periosteal flap treated in this way is then attached over the lesion in the articular surface of the knee joint by a sewing procedure, for example using resolvable material. The joint is then closed. The joint is injected with a solution containing CDMPs dissolved or suspended in a pharmacologically acceptable carrier to maintain the chondrogenic process. Injection is continued until the monitoring physician indicates repair of the cartilage is complete. The patient notices markedly less joint pain as the cartilage repair process progresses. Exam by arthroscopy indicates repair of the lesion within several weeks following the initial procedure.

We also contemplate gene therapy protocols based on expression of CDMP cDNAs or genomic constructs as a way of facilitating *in vivo* cartilage repair. Diseases such as chondromalacia or osteoarthritis are examples for which such gene therapy protocols are contemplated. Therapy may be achieved by genetically altering synoviocytes, periosteal cells or chondrocytes by transfection or infection with recombinant constructs that direct expression of the CDMPs. Such altered cells can then be returned to the joint cavity. We contemplate that gene transfer can be accomplished by retroviral, adenoviral, herpesvirus and adeno associated virus vectors.

Both *in vivo* and *ex vivo* approaches are anticipated for continuously delivering CDMPs for the purpose of retarding ongoing osteoarthritic processes and for promoting cartilage repair and regeneration. In addition, one might employ inducible promoter constructs (e.g. under transcriptional control of a dexamethasone promoter) in gene therapy applications of the present invention. A combined approach to osteoarthritis therapy may have particular advantages. For example, CDMP-2 could be continuously expressed to support the integrity of the articular surface. An inducible construct could be employed to express CDMP-1 so that chondrogenesis could be accelerated at the time of more aggressive destruction.

The foregoing experimental results and characterization confirmed the CDMP-1 and CDMP-2 isolates belong to the TGF-β superfamily. Based on the high percentage identity of their C-terminal domains, CDMP-1 and CDMP-2 can be classified as members of a novel subfamily. Although CDMP-1 and CDMP-2 were identified in two different species (human and bovine), they repres nt distinct genes since the sequences of their pro-regions are significantly divergent.

Several BMPs have now been implicated in early skeletal development, including BMPs -2, -4, -5, -7 and CDMP-1 (GDF-5). Other members, such as BMPs -3, -6, -7 and CDMP-2, may be involved in later stages of skeletal formation (13, 15). The role of the BMPs in early development could be chemotactic, mitogenic or inductive. Their function in later stages of skeletal development might be promotion of differentiation and maintenance of the established phenotype. The availability of mouse strains with null mutations in specific BMP members, such as the short-ear mice (Bmp5) and the bp mice (Cdmp1/Gdf5), allows analysis of the specific contributions of the respective members in each of the stages of skeletal development.

The absence of expression of both CDMP-1 and CDMP-2 in the axial skeleton has implications for models of skeletal development. For example, the *bp* mice have disturbed limb development but a normal axial skeleton. This is the first evidence that the developmental mechanisms and differentiation pathways of the vertebral bodies are distinct from those of the peripheral skeletal elements. Further, this indicates the basic form and pattern of the skeleton are likely to be determined by a number of BMP-like signaling molecules.

WE CLAIM:

1. A purified cartilage extract that stimulates local cartilage formation when combined with a matrix and implanted into a mammal, said extract being produced by a method comprising:

(a) obtaining cartilage tissue;

(b) homogenizing said cartilage tissue in the presence of chaotropic agents under conditions that permit separation of proteins from proteoglycans;

(c) separating said proteins from said proteoglycans; and

(d) obtaining said proteins.

2. The extract of Claim 1, wherein said extract is obtained by a method in which step (c) comprises use of a sepharose column.

3. The extract of Claim 1, wherein said extract is obtained by a method which additionally comprises the steps of separating said proteins on a molecular sieve and obtaining those proteins having a molecular weight between 30 kDa and 60 kDa.

4. The extract of Claim 1, wherein said extract is an extract of articular cartilage.

5. The extract of Claim 1, wherein said extract is an extract of epiphyseal cartilage.

6. A method of preparing a partially purified articular cartilage extract having chondrogenic activity, comprising the steps:

(a) obtaining cartilage tissue;

(b) homogenizing said cartilage tissue in the presence of chaotropic agents under conditions that permit separation of proteins from proteoglycans;

(c) separating said proteins from said proteoglycans; and

(d) obtaining said proteins.

7. The method of Claim 6, wherein step (c) comprises use of a sepharose column.

8. The method of Claim 7, wherein step (c) comprises isolating the proteins that bind heparin Sepharose in the presence of 0.15 M NaCl but not in the presence of 1 M NaCl.

9. The method of Claim 6, additionally comprising the steps of separating said proteins on a molecular sieve and obtaining those proteins having a molecular weight between 30 kDa and 60 kDa.

10. An isolated DNA molecule encoding a protein having chondrogenic activity *in vivo* but substantially no osteogenic activity *in vivo*, said molecule having a nucleotide sequence that can hybridize to a polynucleotide having a nucleotide sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12 at 55°C with 0.4× SSC and 0.1% SDS.

11. The isolated DNA molecule of Claim 10, wherein said molecule has a nucleotide sequence selected from the group consisting of SEQ ID NO:13 and SEQ ID NO:14.

12. A recombinant protein having chondrogenic activity *in vivo* but substantially no osteogenic activity *in vivo*, wherein said protein has an amino acid sequence selected from the group consisting of SEQ ID NO:13 and SEQ ID NO:14.

13. A method of stimulating cartilage formation in a mammal, comprising the steps:

a) supplying cartilage-derived morphogenetic proteins having *in vivo* chondrogenic activity;

b) mixing said partially purified proteins with a matrix to produce a product that facilitates administration of said partially purified proteins; and

c) implanting the product of step (b) into the body of mammal to stimulate cartilage formation at the site of implantation.

14. The method of Claim 13, wherein said partially purified cartilage-derived morphogenetic proteins are obtained from articular cartilage or epiphyseal cartilage.

15. The method of Claim 13, wherein the matrix of step (b) comprises a cellular material.

16. The method of Claim 13, wherein mixing step (b) additionally comprises mixing of viable chondroblast or chondrocytes.

17. The method of Claim 5, wherein the implanting step comprises implanting subcutaneously.

18. The method of Claim 5, wherein the implanting step compises implanting subcutaneously.

19. The method of Claim 5, wherein the implanting step comprises implanting intramuscularly.

20. A composition that can be administered to a mammal for the purpose of stimulating chondrogenic activity at the site of administration without substantially stimulating osteogenic activity, said composition comprising at least one cartilage-derived morphogenetic protein and a matrix.

21. The composition of Claim 20, wherein said cartilage-derived morphogenetic protein is derived from an extract of cartilage tissue.

22. The composition of Claim 20, wherein said cartilage tissue is selected from the group consisting of articular cartilage and epiphyseal cartilage.

23. The composition of Claim 20, wherein said cartilage-derived morphogenetic protein is a recombinant protein.

24. The composition of Claim 20, wherein said recombinant protein has a sequence selected from the group consisting of SEQ ID NO:13 and SEQ ID NO:14.

25. The composition of Claim 20, wherein said matrix is selected from the group consisting of fibrin glue, freeze-dried cartilage, collagens and guanidinium-insoluble collagenous residue of demineralized bone.

26. The composition of Claim 20, wherein said matrix is a non-resorbable matrix selected from the group comprising tetracalcium phosphate and hydroxyapatite.

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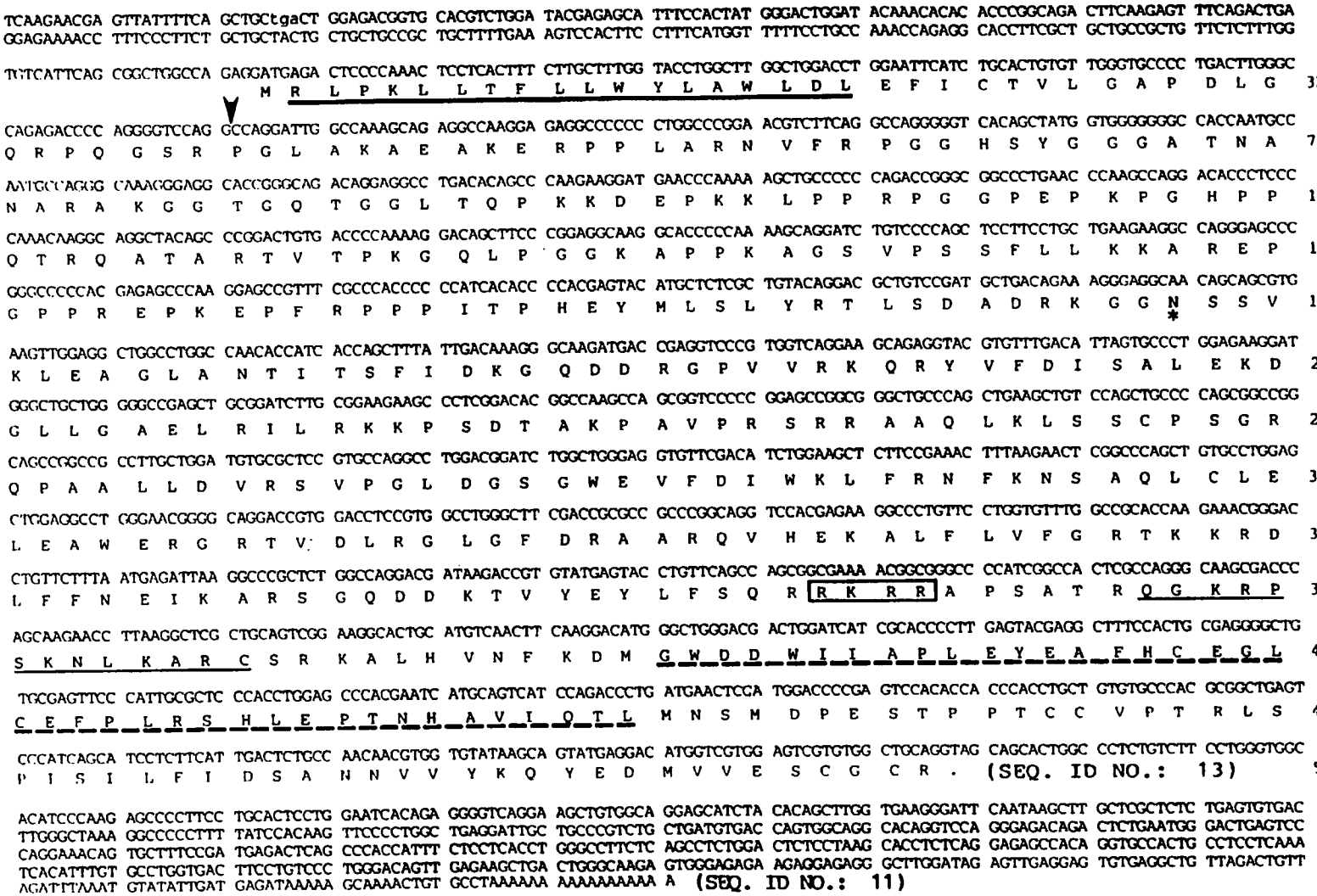

FIGURE 1

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CGAGCTCCG CCGAGTGGG CTCGGCCAAG GGAATGCGAA CGCGCAAGGA AGGAAGGATG CCGCGGGCGC CGAGAGAGAA TGCCACGGCC CGGGAGCCCC TGGATCGCCA GGAGCCCCCG 120
R A S A E L G S A K G M R T R K E G R M P R A P R E N A T A R E P L D R Q E P P
CCCAGGCCGC AGGAGAGCC CCAGCGGCGG CCGCCAGAGC AGCCTGAAGC TCGGGAGCCT CCCGGCAGGG GCCCCGCGTT GGTGCCCCAC GAGTACATGC TGTCAATCTA CAGGACTTAC 240
P R P Q E E P Q R R P P Q Q P E A R E P P G R G P R L V P H E Y H L S I Y R T Y
TCCATCGCCG AGAAGCTGGG CATCAATGCT AGCTTTTTCC AGTCTTCCAA GTCGGCTAAT ACGATCACTA GCTTTGTAGA CAGGGGACTA GACGATCTCT CGCACACTCC TCTCCGGAGA 360
S I A E K L G I N A S F F Q S S K S A N T I T S F V D R G L D D L S H T P L R R
CAGAACTATT TGTTTGATGT GTCCACGCTC TCAGACAAAG AAGAGCTGGT GGGCGCGGAC GTGCGGCTGT TTCGCCAGGC GCCCGCTGCC CTGGCGCCGC CGGCGGCCGC TCCGCTTGCA 480
Q K Y L F D V S T L S D K E E L V G A D V R L F R Q A P A A A L A P P A A A P L A
GCTCTTCGCC TGCCAGTCGC CCCTGCTGCT GGAAGCGCGG AGCCTGGACC CGCAGGGGCG CCCCGGCCCG GCTGGGAAGT CTTCGACGTG TGGCGGGGCC TGCGCCCCCA GCCCTGGAAG 600
A L R L P V A P A A G S A E P G P A G A P R P G W E V F D V W R G L R P Q P W K
CAGCTGTGCT TGGAGCTTCG GGCCGCCGTGG GGCGGCGCGC CGGGCGCCGC GGAGGACGAG GCGCCGCACGC CTGGGCCCCA GCAGCCGCCG CCCCCGGACC TGCGGAGTCT GGGCTTCGGC 720
Q L C L E L R A A W G G E P G A A E D E A R T P G P Q Q P P P D L R S L G F G
CGCAGGGTGC GGACCCCCCA GGAGCGCGCC TTGCTCGTCG TGTTCTCCAG GTCCCAGCGC AAGACCCTGT TCGCCGAGAT GCGCGAGCAG CTGGGCTCGG CGACCGAGGT GGTCGGCCCC 840
R R V R T P Q E R A L L V V F S R S Q R K T L F A E H R E Q L G S A T E V V G P
GGTGGTGGGG CCGAGGGGTC GGGCCGCCGG CGCCGCCGCC GCCGTCGGGC ACCCCGGACG CTGGGCTCTG GTCGCCCTCG CCTGGCCGGC GGCGGCCCAC GGCCTTCGCC 960
G G G A E G S G P P P P P P P S G T P D A G L W S P S P G R R R T A F A
AGCCGCCACG GCAAGCGGCA CGGCAAGAAG TCGAGGCTGC GCTGCAGCAA GAAGCCCCTG CACGTGAACT TCAAGGAGCT GGGCTGGGAC GACTGGATTA TCGCGCCCCT GGAGTACGAG 1080
S R H G K R H G K K S R L R C S K K P L H V N F K E L G W Q D W I I A P L E Y E
GCCTACCACT GCGAGGGCGT GTGCGACTTC CCGCTACCGT CGCACCTGGA GCCCACCAAC CACGCCATCA TCCAGACGCT GATGAACTCC ATGGACCCCG GCTCCACCCC GCCCAGCTGC 1200
A Y H C E G V C D F P L R S H L E P T N H A I I Q T L
TGCCTGCCCA CCAAATTGAC TCCCATCAGC ATCTTGTACA TCGACGCGGG CAATAATGTG GTCTACAACG AGTACGAGGA GATGGTGGTG GAGTCGTGCG GCTGCAGG 1308
C V P T K L T F I S I L Y I D A G N N V V Y N E Y E E H V V E S C G C R
```

FIGURE 2

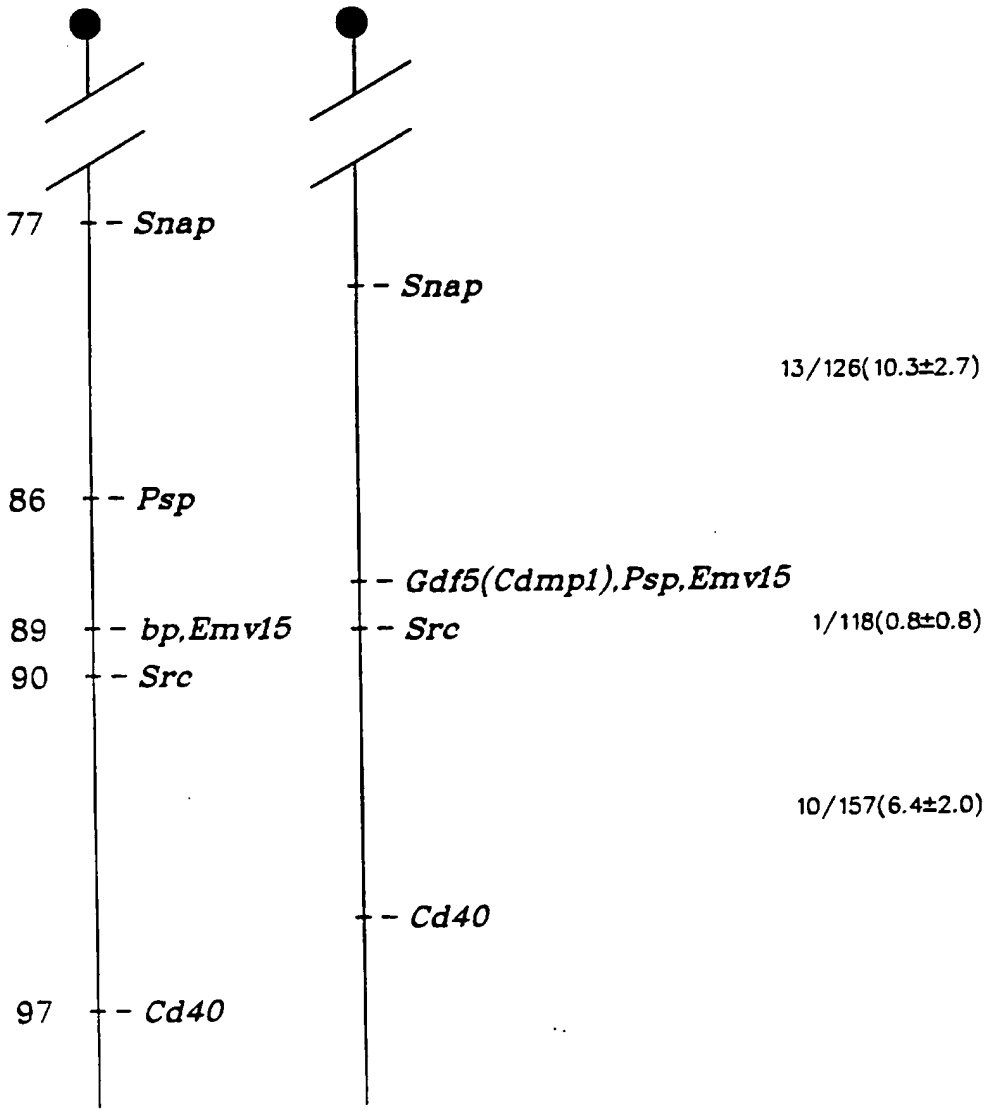
77 - Snap
- Snap
13/126(10.3±2.7)
86 - Psp
- Gdf5(Cdmp1),Psp,Emv15
89 - bp,Emv15
- Src
1/118(0.8±0.8)
90 - Src
10/157(6.4±2.0)
- Cd40
97 - Cd40

FIG. 3

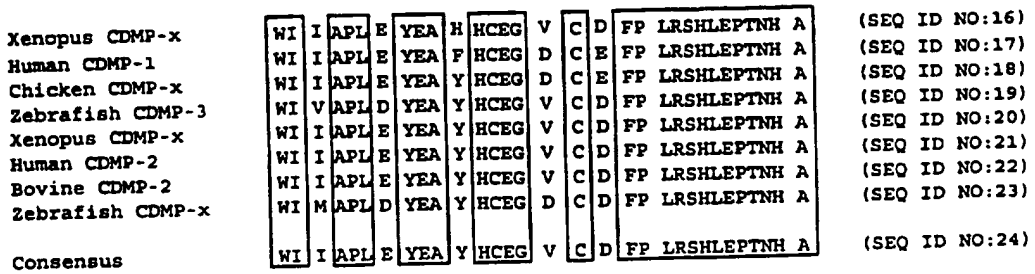
Xenopus CDMP-x
Human CDMP-1
Chicken CDMP-x
Zebrafish CDMP-3
Xenopus CDMP-x
Human CDMP-2
Bovine CDMP-2
Zebrafish CDMP-x
Consensus
WI I APL E YEA H HCEG V C D FP LRSHLEPTNH A (SEQ ID NO:16)
WI I APL E YEA F HCEG D C E FP LRSHLEPTNH A (SEQ ID NO:17)
WI I APL E YEA Y HCEG D C E FP LRSHLEPTNH A (SEQ ID NO:18)
WI V APL D YEA Y HCEG V C D FP LRSHLEPTNH A (SEQ ID NO:19)
WI I APL E YEA Y HCEG V C D FP LRSHLEPTNH A (SEQ ID NO:20)
WI I APL E YEA Y HCEG V C D FP LRSHLEPTNH A (SEQ ID NO:21)
WI I APL E YEA Y HCEG V C D FP LRSHLEPTNH A (SEQ ID NO:22)
WI M APL D YEA Y HCEG D C D FP LRSHLEPTNH A (SEQ ID NO:23)
WI I APL E YEA Y HCEG V C D FP LRSHLEPTNH A (SEQ ID NO:24)

FIGURE 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12814

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C07K 14/51; C12N 15/12; A61F 13/00; A61K 38/18
US CL :424/422; 514/2, 21; 530/350, 415, 417, 840; 536/23.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/422; 514/2, 21; 530/350, 415, 417, 840; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, BIOSIS, CA, INPADOC, JICST-E, MEDLINE search terms: cartilage morphogen or induc, chrondrogen, protein or peptide or polypeptide or factor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,E	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 269, Number 45, issued 11 November 1994, Chang et al, "Cartilage-derived Morphogenetic Proteins", pages 28227-28234, see entire document.	1-26
A	US, A, 4,935,497 (VEIS ET AL) 19 June 1990, see abstract.	1
X ---- Y	US, A, 4,810,691 (SEYEDIN ET AL) 07 March 1989, see column 2, line 28 - column 3, line 5, and column 9, line 44 - column 10, line 49.	1,2,4,5,13, 15-20, 23 ---------- 14,21,22,25,26

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
10 FEBRUARY 1995

Date of mailing of the international search report
16 MAR 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
STEPHEN WALSH
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12814

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,609,551 (CAPLAN ET AL) 02 September 1986, see column 1, line 35 - column 2, line 41.	13-26
Y	NATURE, Volume 368, issued 14 April 1994, Storm et al, "Limb alterations in brachypodism mice due to mutations in a new member of the TGFβ-superfamily", pages 639-643, see entire document.	1-26
Y	SCIENCE, Volume 242, issued 16 December 1988, Wozney et al, "Novel Regulators of Bone Formation: Molecular Clones and Activities", pages 1528-1534, see Table 1 at page 1531.	6-9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12814

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12814

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-9 and 12-26, drawn to a purified cartilage extract, a method of preparing a cartilage extract, a recombinant protein, a method of stimulating cartilage formation in a mammal, and a composition that can be administered to a mammal, classified in Class 514, subclass 2.

II. Claims 10 and 11, drawn to an isolated DNA molecule encoding a protein, classified in Class 536, subclass 23.5.

Invention I is drawn to protein compositions of matter that are materially different from the DNA compositions of Invention II because the special technical features which define proteins distinguish them from DNA. The recombinant protein of I is indistinguishable from the same protein isolated from the cartilage extract by the method in Invention I. The DNA of Invention II cannot be used in or produced by the methods of Invention I. The claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.